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Stimulation of growth hormone by kisspeptin antagonists in ewes

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Abstract

Kisspeptin signalling is indispensable for fertility, stimulating gonadotropin-releasing hormone (GnRH) secretion and mediating gonadal steroid feedback on GnRH neurons. Moreover, kisspeptin neurons have been implicated in other non-reproductive neuroendocrine roles. Kisspeptin appears to also regulate growth hormone secretion but much of the data appear contradictory. We sought to clarify a potential role of kisspeptin in GH regulation by examining the effect of kisspeptin antagonists on growth hormone (GH) secretion in ewes under various physiological conditions. Our data show clear and robust increases in GH secretion following lateral ventricle or third ventricle infusion of kisspeptin antagonists p-234 and p-271 in either ovariectomised or anestrous ewes. Central infusion of kisspeptin-10 had no effect on GH secretion. To determine the level at which kisspeptin may influence GH secretion we examined expression of the cognate kisspeptin receptor, GPR54, in pituitary cells and showed by immunocytochemistry that the majority of somatotropes express GPR54 while expression was largely negative in other pituitary cells. Overall, we have demonstrated that blocking kisspeptin signalling by antagonists stimulates GH secretion in ewes and that this is likely mediated by inhibiting endogenous kisspeptin activation of GPR54 expressed on somatotropes. The findings suggest that endogenous kisspeptin inhibits growth hormone secretion through GPR54 expressed on somatotropes.

Introduction

Kisspeptin and its cognate receptor GPR54 are now well accepted as a critical component of central nervous system regulation of GnRH neuron control of reproductive hormones (de Roux, et al. 2003; Seminara, et al. 2003; Topaloglu, et al. 2012). Kisspeptin neuron activity is essential for the stimulation of GnRH secretion (Gottsch, et al. 2004; Han, et al. 2005) and mediates both negative and positive feedback regulation by gonadal steroid hormones onto GnRH neurons (Smith 2013) and kisspeptin signalling is required for the onset of puberty (Han et al. 2005).

Leptin and products of metabolism also impact on kisspeptin neuron function and kisspeptin neurons are hypothesized to be “whole body sensors” linking physiological status to reproduction (Pineda, et al. 2010a). Growth hormone (GH) plays a role in normal reproductive function (Hull and Harvey 2001, 2002) and in metabolic regulation. A substantial number of studies have now reported effects of kisspeptin on GH secretion in various species, under different physiological conditions, using peripheral or central administration, and by examining direct effects on pituitary cells in culture (Foradori, et al. 2017; Gutierrez-Pascual, et al. 2007; Kadokawa, et al. 2008; Kadokawa, et al. 2007; Lents, et al. 2008; Whitlock, et al. 2010; Whitlock, et al. 2008). Kisspeptin is a member of the large family of RF-amide peptides which target an equally large family of cognate receptors (Dockray 2004). Consequently doses and mode of administration of kisspeptin might have effects that are not mediated via the GPR54 cognate receptor but result from activation of other GPCRs (Oishi, et al. 2011), which may contribute to contradictory reports of kisspeptin effects on GH.

In an attempt to clarify effects of kisspeptin on GH we have studied the effects of kisspeptin antagonists on GH secretion in ewes under various physiological conditions. Our data suggest that GH is under endogenous kisspeptin inhibition, which is relieved by kisspeptin antagonist treatment, resulting in an elevation of GH.

Materials and Methods

Animals and Peptides

All experimental procedures were conducted under a protocol approved by the Monash School of Biomedical Sciences Animal Ethics Committee. Adult Corriedale ewes were housed under natural lighting. Kisspeptin antagonist (peptide-234 and peptide-271) were synthesized by EZBiolab Inc. (Carmel, IN).

The peptide sequence of peptide-234 is ac-(D-A)NWNGFG(D-W)RF-NH₂ (Roseweir, et al. 2009). Peptide-271 is the same sequence as peptide-234 with the addition of a seven amino acid cationic cell-penetrating peptide penetratin RRMKWKK extension at the NH₂ terminus of peptide-234. This peptide was shown *in vitro* and *in vivo* in rats, mice, sheep and monkeys to be an effective kisspeptin antagonist (Albers-Wolthers, et al. 2017; De Bond, et al. 2013; Guerriero, et al. 2012; Millar, et al. 2010; Pineda, et al. 2010b; Roseweir et al. 2009; Roseweir and Millar 2013; Smith, et al. 2011).

Kisspeptin peptide YNWNSFGLRY-NH₂ corresponding to the murine C terminal Kiss1 decapeptide was obtained from Phoenix Pharmaceuticals Ltd. (Belmont, CA). This sequence is identical to the C-terminal sequence of ovine kisspeptin (GenBank accession No. DQ059506).

Effect of kisspeptin antagonists on GH secretion in ewes

Experiment 1: Effect of third ventricular infusion of peptide 234 on GH in ovariectomized ewes.

Experiments were performed as described previously (Roseweir et al. 2009). Animals were bilaterally ovariectomized (OVX) at least one month before any experimental manipulations. Permanent indwelling third cerebral ventricular (3V) cannulae were implanted in a subsequent surgical procedure as described previously (Barker-Gibb, et al. 1995). Approximately 2 weeks after 3 V surgery, one external jugular vein was cannulated for blood sampling and animals housed in single pens; cannulae were kept patent with heparinized saline. Ewes were assigned to treatment groups (n=4 per group); peptide 234 (diluted in aCSF; 150 mM NaCl, 1.2 mM CaCl₂, 1 mM MgCl₂, 2.8 mM KCl) or control (aCSF only). The following

day, infusion lines were connected to 3V cannulae, and blood sampling commenced at 7:00 am. Samples were collected every 10 min. After 3 h of sampling, peptide 234 (or control) was infused into the 3V at a dose of 40 µg/h for 1 h, with an initial dose of 10µg. Both peptide 234 and vehicle were infused at 200 µl/h using Graseby MS16A infusion pumps (Smith Medical Australasia). After infusion, 3 V lines remained in place, and blood sampling continued for a further 2 h (total of 6 h). Plasma was harvested immediately from samples and frozen at -20°C until assayed.

Experiment 2: Effect of lateral ventricular infusion of peptide 271 on GH in ovariectomized ewes.

Experiments were performed as previously described (Smith et al. 2011). Ewes were bilaterally OVX as above and permanent indwelling lateral ventricle (LV) cannulae were implanted as described previously (Henry et al., 2008). Experiments procedures and blood sampling were similar to above ewes received either peptide 271 (1 h continuous infusion 300 µg/h, with an initial 200 µg/h loading dose; n=5) or vehicle (aCSF; n=5) into the LV (200 µg/h). After infusion, LV lines remained in place as blood sampling continued for 3 h. Plasma was harvested immediately and frozen at -20°C until assayed.

Experiment 3: Effect of long-term lateral ventricular infusion of peptide 271 on GH in ovary intact estrogen treated anestrus ewes.

Experiments were performed as previously described (Smith et al. 2011), in order to determine the role of kisspeptin signalling in mediating the LH surge, hence a longer infusion period of kisspeptin antagonist was employed. LV and jugular vein cannulae were implanted (as above) in anestrus ewes. All ewes received an initial intramuscular (im) injection of 50 µg estradiol benzoate (Intervet, New South Wales, Australia) in 1 ml peanut oil. Blood sampling commenced 9 h later and samples (5 ml) were taken every 10 min for 9 h, then every 30 min for a further 12h. After 3 h of blood sampling, ewes received LV infusions (200 µl/h) of kisspeptin antagonist (8 h continuous infusion 300 µg/h, with an initial 200 µg loading dose; n=6) or vehicle (aCSF; n=6). Plasma was harvested immediately and frozen at -20°C until assayed.

Experiment 4: Effect of lateral ventricular infusion of kisspeptin-10 on GH secretion in ovary-intact anestrus ewes.

Experiments were performed as previously described (Li, et al. 2015), in order to determine the role of kisspeptin in mediating the LH pulses. Ovary intact ewes during the southern hemisphere anestrus season (September) were prepared for LV and jugular vein cannulation (as above). Blood samples were collected every 10 min for 3 h then animals received kisspeptin (40 µg/h with an initial loading dose of 40 µg, n=6) or vehicle (aCSF, n=6) treatment for 4 h (200 µl/h). After the infusion, LV lines remained in place, and blood sampling continued for a further 2 h (total of 9 h). Plasma was harvested immediately from samples and frozen at -20 C until assayed.

GH Radioimmunoassay

Plasma samples from ewes were assayed in duplicate following the method of Thomas et al. (Thomas, et al. 1990) using the standard NIDDK-oGH-I-4 and NIDDK-anti-oGH-2 antiserum. The assay sensitivity was 1 ng/ml, the intra-assay CV was less than 10% between 4 and 51 ng/ml and the interassay CV was 20%.

Immunocytochemistry of GPR54 and pituitary hormones in ovine pituitary

An antiserum (RM1211) to the carboxyl terminal sequence CVLGEDNAPL of human GPR54 conjugated to haemocyanin was raised in rabbits. The specificity of the highest titre antiserum was demonstrated by positive membrane staining in COS7 cells transfected with the human GPR54 pcDNA and an absence of staining in untransfected COS7 cells (data not shown). Pituitary glands from adult ewes were fixed in Bouin's or 4% Neutral Buffered formaldehyde (4%NBF) and sections processed for immunocytochemistry using GPR54 antiserum and counter stained with hematoxylin. To determine which pituitary cell types expressed GPR54, dual staining was conducted with rabbit antisera raised against ovine prolactin, growth hormone, luteinizing hormone and adreno-corticotrophic hormone

(supplied by the National Hormone and Pituitary Programme of NIDDK). Following dewaxing and rehydration of sections through graded ethanols endogenous peroxidase activity was blocked using 3% hydrogen peroxide in methanol, before blocking endogenous biotin using avidin/biotin block (Vector labs, UK). Sections were then incubated with rabbit anti GPR54 antiserum in 20% normal goat serum in Tris Buffered Saline (TBS). Binding was detected using goat anti rabbit biotinylated fab (Abcam, UK) and Streptavidin-HRP before visualising with Diaminobenzidine (DAB). Following a repeat of the avidin/blocking, sections were incubated with rabbit antiserum to either porcine ACTH, ovine GH, ovine LH-beta or ovine prolactin at NIDDK recommended dilutions. Binding was detected using secondary biotinylated fab antibody (Abcam, UK) and Streptavidin-AP before visualising with fast blue (Abcam).

Data Analysis

All group data are presented as the mean \pm SEM. Plasma GH concentrations in response to treatment over time were examined by repeated measures ANOVA. The effect of kisspeptin antagonist or kisspeptin treatment on GH was further examined by comparing the mean GH concentration in samples pre-infusion (0-180 min), during the infusion (Experiment 1 and 2, 180-240 min; Experiment 3, 180-720 min; Experiment 4, 180-420 min) and post infusion (Experiment 1, 240-360 min; Experiment 2, 240-420 min; Experiment 3, 720-1260 min; Experiment 4, 420-540 min). The mean GH values were calculated for each animal during the relevant time phase and used to generate group means. Data were assessed by repeated measures ANOVA.

Results

Effect of kisspeptin antagonist on GH secretion in ewes

Third ventricular administration of kisspeptin antagonist peptide-234 significantly increased the plasma concentration of GH in OVX ewes (Figure 1a). Mean GH was 3-fold greater during the peptide-234 infusion compared to aCSF control (Figure 1b, $P < 0.005$). Table 1 provides a summary of the effects of peptide-234 on GH and our previously published analysis of plasma LH, prolactin and cortisol in ovariectomised ewes (Roseweir et al. 2009).

Lateral ventricular administration of peptide-271 resulted in similar effects (Figure 2a). Mean GH levels (Figure 2b) were again significantly elevated during the time of the antagonist infusion compared to vehicle controls ($P < 0.01$). Previously, peptide-271 inhibited LH pulses and mean LH in ovariectomised ewes (Smith et al. 2011).

Similar results were achieved with central peptide-271 treatment in ovary intact ewes (Figure 3a). Here, the mean GH concentration over the 8 h continuous peptide-271 infusion was significantly greater than during the aCSF infusion ($P < 0.001$, Figure 3b). From our previous analysis, the peptide-271 kisspeptin antagonist in this paradigm significantly attenuated the estradiol-induced LH surge (Smith et al. 2011).

Effect of kisspeptin-10 on GH secretion in ovary-intact anestrous ewes

Lateral ventricular administration of kisspeptin-10 had no effect on plasma GH concentrations in ovary-intact anestrous ewes (Figure 4a). Mean GH concentration over the 4 h continuous infusion period was similar between kisspeptin-10 and control ewes (Figure 4b). In the same animals, there was a significant stimulation of LH (Li et al. 2015).

Ovine somatotropes co-express kisspeptin receptor

Pituitaries from adult ewes displayed clear immunostaining with the GPR54 antiserum RM 1211 in both NBF (Fig 5E) and Bouins (Fig 5F) fixation. Staining was ablated by preincubation of the antiserum with the CVLGEDNAPL immunogen. (data not shown). Subsequent staining with rabbit antisera to prolactin, adreno-corticotropin, luteinizing hormone and growth hormone in Bouins clearly revealed the various cell types but staining of some of these hormones was not clear with NBF staining (data not shown). In the Bouins fixed tissue there was little or no co-localisation of staining (<1%) in corticotropes (Fig 5A), gonadotropes (Fig 5C), and lactotropes (<1%, Fig 5D), while the majority of somatotropes (86%, Fig 5B) stained positive for GPR54.

Discussion

Adequate GH secretion is required for optimal reproductive hormone production during puberty and in adulthood. Because GH is involved in metabolic regulation and recent evidence supports the concept that kisspeptin is an integrator of nutrition and metabolic regulation of reproduction (De Bond and Smith 2014; Pineda et al. 2010a) it is plausible that kisspeptin may be a regulator of GH. A number of publications support this notion. Kisspeptin stimulated calcium influx in rat somatotropes and secretion of GH from rat (Gutierrez-Pascual et al. 2007) and bovine (Kadokawa et al. 2007) pituitary cells. However, Jayasena et al (Jayasena, et al. 2014) found no effect of kisspeptin on GH secretion from the pituitary *in vitro*. *In vivo* studies present a complexity of findings including contradictory reports. High iv doses ($3 > \text{nmol/kg}$) of kisspeptin stimulated prolonged GH release in prepubertal heifers (Kadokawa et al. 2008) but a lower dose of kisspeptin had no effect in ovariectomised adult cows but stimulated GH when progesterone or estrogen or both were administered (Whitlock et al. 2008). Central or systemic administration of kisspeptin had no effect on GH in prepubertal female pigs while inducing LH secretion (Lents et al. 2008). Similarly kisspeptin peripheral infusion had no effect on GH in cows while robustly stimulating LH but preinfusion of kisspeptin reduced GH responses to GHRH and somatostatin withdrawal (Whitlock et al. 2010). This study also reported no GH response to iv kisspeptin in ewes but a small response when administered icv (Whitlock et al. 2010). However, recently published data show

kisspeptin can stimulate GH, but only in fasted ewes, by a ghrelin-NPY mediated mechanism (Foradori et al. 2017). These complex, and often, contradictory reports may be a result of the large family of RFamide receptors, which can be targeted by kisspeptin. We attempted to clarify the field by utilising specific antagonists to GPR54. In our study we found no effect of administration of kisspeptin into the LV on GH secretion in intact ewes, which is in agreement with the reported lack of response to peripheral administration in intact cows studies (Whitlock et al. 2008) and prepubertal female pigs (Lents et al. 2008) but contrasts with a small GH increase reported in ewes (Whitlock et al. 2010) although the immediate nutritional status may be key in this model (Foradori et al. 2017). In women, both acute and chronic peripheral administration of kisspeptin-54 failed to alter GH concentrations (Jayasena et al. 2014). As mentioned above, these varying and contradictory reports on the effects of kisspeptin on GH may be the result of cross signalling through the large family of RFamide receptors, especially when high doses are administered centrally. For example we have observed that kisspeptin has a high affinity for the GnIH receptor, GPR147, while GnIH binds poorly to GPR54 (*Millar RP, unpublished data*). In the same study peptide-234 and peptide-271 did not bind to GPR147 suggesting that they do not interact with other RFamide receptors and are better probes for determining the role of kisspeptin in GH secretion.

We have therefore attempted to reconcile these apparently conflicting effects of kisspeptin on GH by administering kisspeptin antagonists in ovariectomised and estrogen treated anestrus ewes. Robust increases in GH were observed in three separate and different experiments after administration of either antagonist peptide-234 or antagonist peptide-271. Moreover, GH was increased regardless of site of administration (3V or LV) and the nature of the antagonist: peptide-271, which has the penetratin sequence attached at the NH₂ terminus, designed to be cell-permeant, or non-cell-permeant peptide-234). For each experiment, ovariectomy or estradiol treatment increased LH pulse and surge dynamics (respectively) and kisspeptin antagonist inhibited plasma LH concentrations but had no effect on cortisol or prolactin (Roseweir et al. 2009; Smith et al. 2011). Thus, the effects are highly specific for GH increase and LH decrease without affecting other pituitary hormones (see Table 1.). The timing of each effect (GH

versus LH) appears, however, to differ, with the relatively delayed effect on LH being due to a time delay in the antagonists having first to compete out endogenous kisspeptin, which then results in a decrease in GnRH followed by a decrease in LH. In contrast, the effect on GH appears much more acute, possibly due to the more direct and immediate effect on the pituitary somatotrope expressing GPR54 receptors.

The stimulation of GH by both kisspeptin antagonists suggests that endogenous kisspeptin is restraining GH secretion in the ewe models we studied. This might be at a hypothalamic level through inhibiting GHRH or stimulating somatostatin as suggested by Whitlock et al (Whitlock et al. 2010), or directly at the pituitary level on somatotropes or through other pituitary cell types that affect the somatotrope. To address the site of kisspeptin action we examined the expression of GPR54 in ewe pituitary cell types using a highly specific antiserum to GPR54. GPR54 was almost exclusively expressed in somatotropes and was virtually absent in LH positive gonadotropes, prolactin positive lactotropes or ACTH positive corticotropes. This expression of GPR54 on somatotropes therefore provides a potential mechanism for the kisspeptin and antagonist effects on GH. GPR54 mRNA expression has been previously reported in dispersed sheep pituitary mainly in somatotropes, with lower expression in enriched lactotropes and gonadotropes (Smith, et al. 2008) but GPR54 protein was not examined as in this study, which showed expression of protein predominantly in somatotropes.

The sources of kisspeptin, which may target GPR54 in somatotropes are potentially through hypothalamic kisspeptin secretion into the portal system or from peripheral tissues via the systemic circulation or through local pituitary cell production. Systemic and portal sources are unlikely because kisspeptin in hypophyseal portal blood in ewes (which is the sum of hypothalamic secretion and systemic levels) is very low with a maximum of 12 pg/ml (Smith et al. 2008). This equates to about 10 pM for Kp-10 and 2 pM for Kp-54. This level is too low for effective receptor occupancy as the affinity (ie 50% receptor occupancy) of GPR54 for kisspeptin is about 1-5 nM (Roseweir et al. 2009) – about 1000 times higher. This indicates that GH stimulation is not from hypothalamic kisspeptin targeting somatotropes via the

portal system or from the general circulation. Expression of kisspeptin has been reported in the pituitary gland of mammalian species (Gutierrez-Pascual et al. 2007; Kotani, et al. 2001; Muir, et al. 2001; Quennell, et al. 2010; Richard, et al. 2008) and in some instances localised to specific pituitary cell types such as corticotropes in the rhesus monkey (Ramaswamy, et al. 2009), gonadotropes in the rat (Richard et al. 2008) and to enriched somatotropes, lactotropes and gonadotropes in ewes (Smith et al. 2008). This therefore suggests that local pituitary kisspeptin production inhibits somatotrope secretion of GH, which is ablated by the antagonists to increase GH.

The demonstration in this study of a virtual absence of GPR54 in gonadotropes in the ewe indicates that previous reports on kisspeptin stimulation of LH secretion from the ewe pituitary in vitro (Smith et al. 2008) are unlikely to be mediated via GPR54 in gonadotropes and are most likely to be indirectly through somatotropes or nonspecific on related RFamine receptors. Importantly, the effect of kisspeptin and/or kisspeptin antagonists on GH release from sheep pituitary primary cultures or cultured somatotropes has not (to our knowledge) been studied. Our demonstration of high expression of GPR54 in somatotropes suggests a possibility that they might secrete activators of gonadotropes to account for the observation of kisspeptin stimulation of LH in the pituitary. It is also feasible that since relatively high doses of kisspeptin were required to stimulate LH from the pituitary in the studies by Witham et al. (Witham, et al. 2013) (100nM) and Gutierrez-Pascual et al. (Gutierrez-Pascual et al. 2007) (10-100nM), the effect may be through kisspeptin activation of other RFamide receptors. Studies using kisspeptin antagonists may clarify this. However the demonstration that kisspeptin at doses that stimulate LH in intact ewes do not stimulate LH when administered to hypothalamic-pituitary-disconnected ewes (Smith et al. 2008) suggest that although kisspeptin can stimulate LH secretion from the pituitary in vitro it does not play a significant role in the direct stimulation of the gonadotrope in the ewe in vivo.

In summary, we have demonstrated that two kisspeptin antagonists administered into the LV or 3V robustly stimulate GH in ovariectomised and estrogen-treated anestrous ewes. The kisspeptin receptor

GPR54 is expressed in the majority of somatotropes suggesting that endogenous kisspeptin binds to these receptors and inhibits GH secretion such that kisspeptin antagonists elevate growth hormone. These findings lay the foundation for more in depth study on the precise physiological relationships between the reproductive and GH systems and the possibility of developing kisspeptin antagonists as therapeutics for GH stimulation.

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418

1 **Figure Legends**

2 Figure 1. Third ventricle infusion of kisspeptin antagonist (KA p-234) stimulates the
 3 secretion of GH in OVX ewes. A, Mean concentrations of GH are shown in ewes treated with
 4 kisspeptin antagonist (*closed squares*) or aCSF (*open circles*). The infusion period is
 5 represented by the closed bar. B, Mean GH pre-, during the infusion, and post-infusion (n=4
 6 per group). A significantly elevated mean GH concentration the during kisspeptin antagonist
 7 infusion was detected. Data are the mean+SEM. ***, P 0.001

8
 9 Figure 2. Lateral ventricle infusion of kisspeptin antagonist (KA p-271) stimulated the
 10 secretion of GH in OVX ewes. A, Mean concentrations of GH are shown in ewes treated with
 11 kisspeptin antagonist (*closed squares*) or aCSF (*open circles*). The infusion period is
 12 represented by the closed bar. B, Mean GH pre-, during the infusion, and post-infusion (n=5
 13 per group). A significantly elevated mean GH concentration the during kisspeptin antagonist
 14 infusion was detected. Data are the mean+SEM. **, P 0.01

15
 16 Figure 3. Long term (8 h) lateral ventricle infusion of kisspeptin antagonist (KA p-271)
 17 stimulated the secretion of GH in intact anestrus ewes. A, Mean concentrations of GH are
 18 shown in ewes treated with kisspeptin antagonist (*closed squares*) or aCSF (*open circles*).
 19 The infusion period is represented by the closed bar. B, Mean GH pre-, during the infusion,
 20 and post-infusion (n=6 per group). A significantly elevated mean GH concentration the
 21 during kisspeptin antagonist infusion was detected. Data are the mean+SEM. ***, P 0.001

22
 23 Figure 4. Central infusion of kisspeptin had no effect on secretory pulses of GH in ewes. A,
 24 Concentrations of GH are shown in ewes treated with kisspeptin or aCSF (vehicle). The

25 infusion period is represented by the closed bar. B, Mean GH pre-, during the infusion, and
26 post-infusion (n = 6 per group). Data are mean+SEM.

27

28 Figure 5. Representative photomicrographs showing immunohistochemical localisation of
29 GPR54 (brown) in the adult ewe pituitary. Immunostaining with rabbit antisera to adreno-
30 corticotropin (A, ACTH + GPR54), growth hormone (B, GH + GPR54), luteinizing hormone
31 (C, LH + GPR54) and prolactin (D, PRI + GPR54) revealed the various cell types (blue). The
32 majority of somatotropes (86%) stained positive for GPR54 (B). E and F show GPR54
33 staining in pituitary sections fixed with 4% Neutral Buffered formaldehyde (E; 205-0198
34 NBF) or Bouins (F; 2005-200 Bouins) and counter stained with hematoxylin (blue). Scale
35 bar, 100 μ m.

36

Figure 1

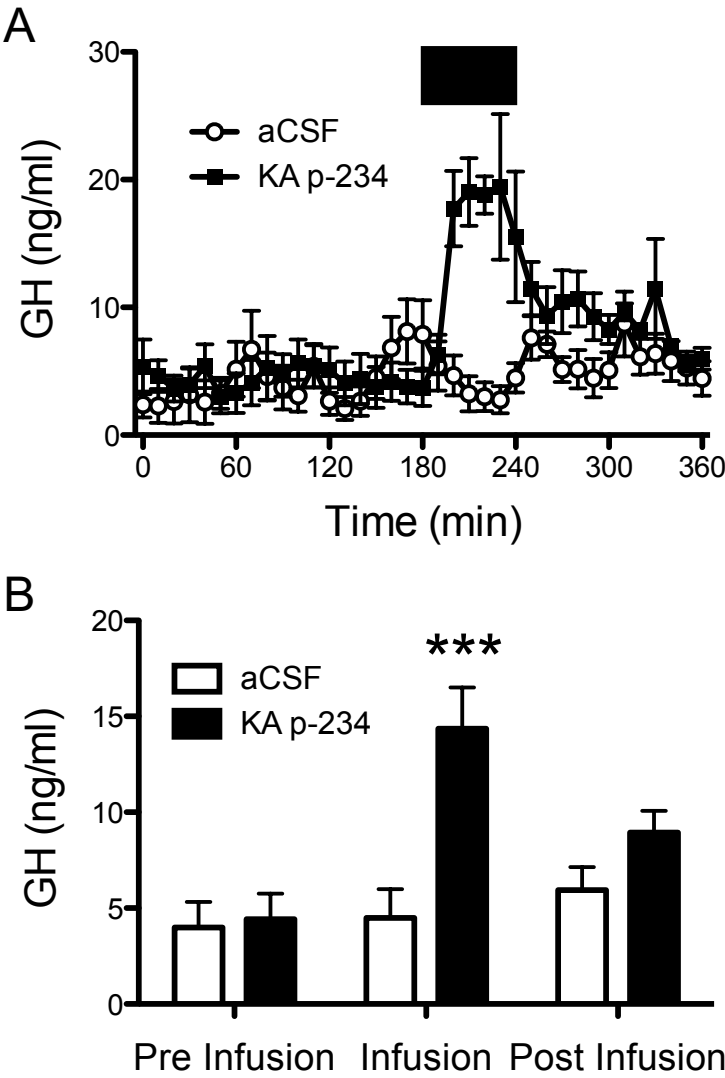


Figure 2

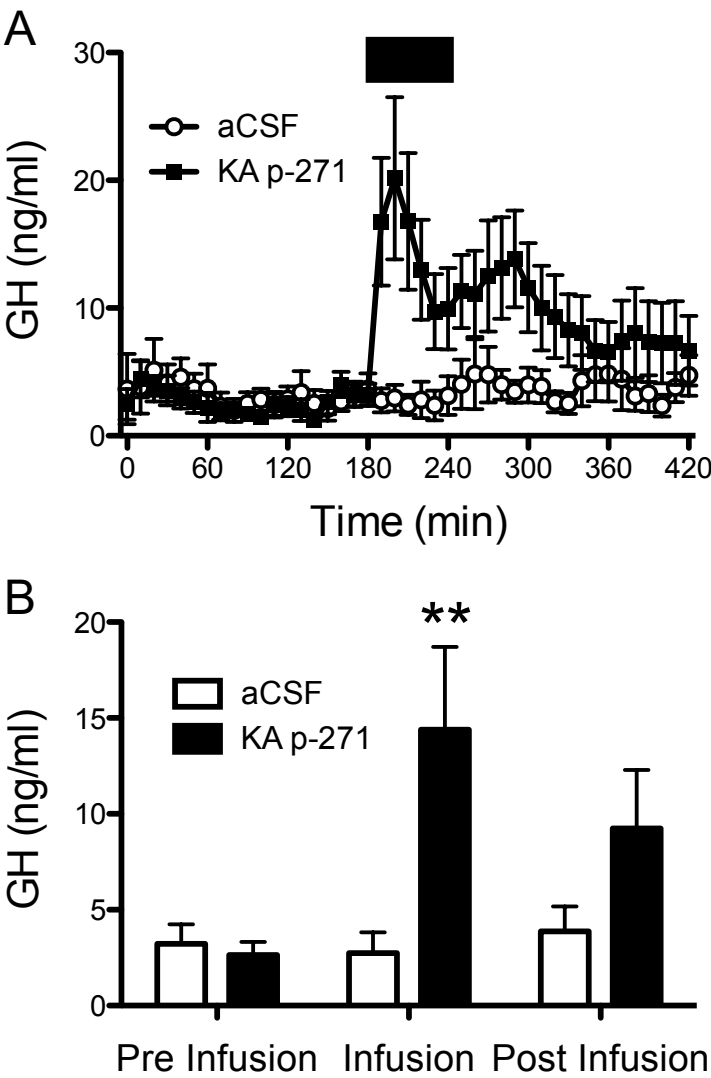


Figure 3

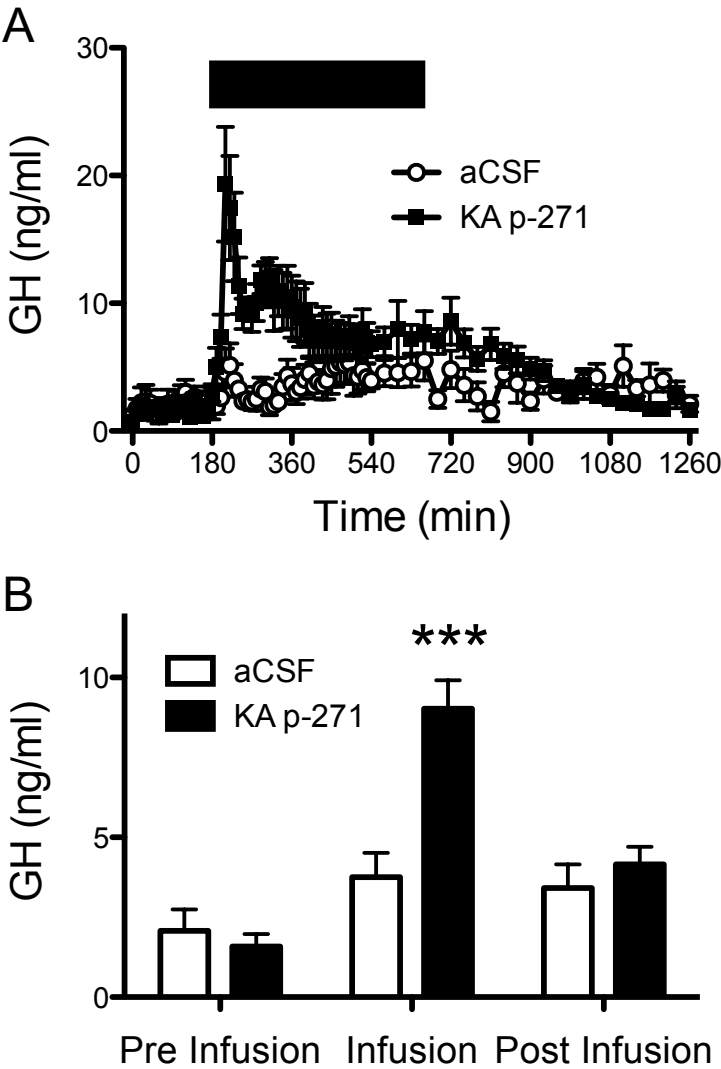
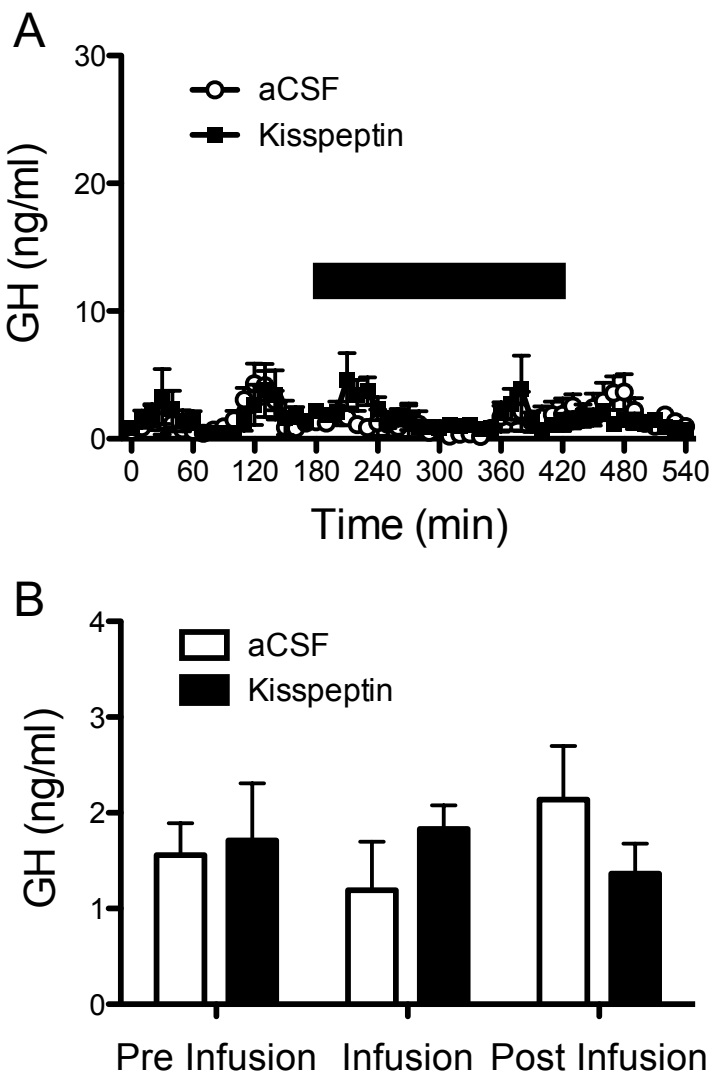
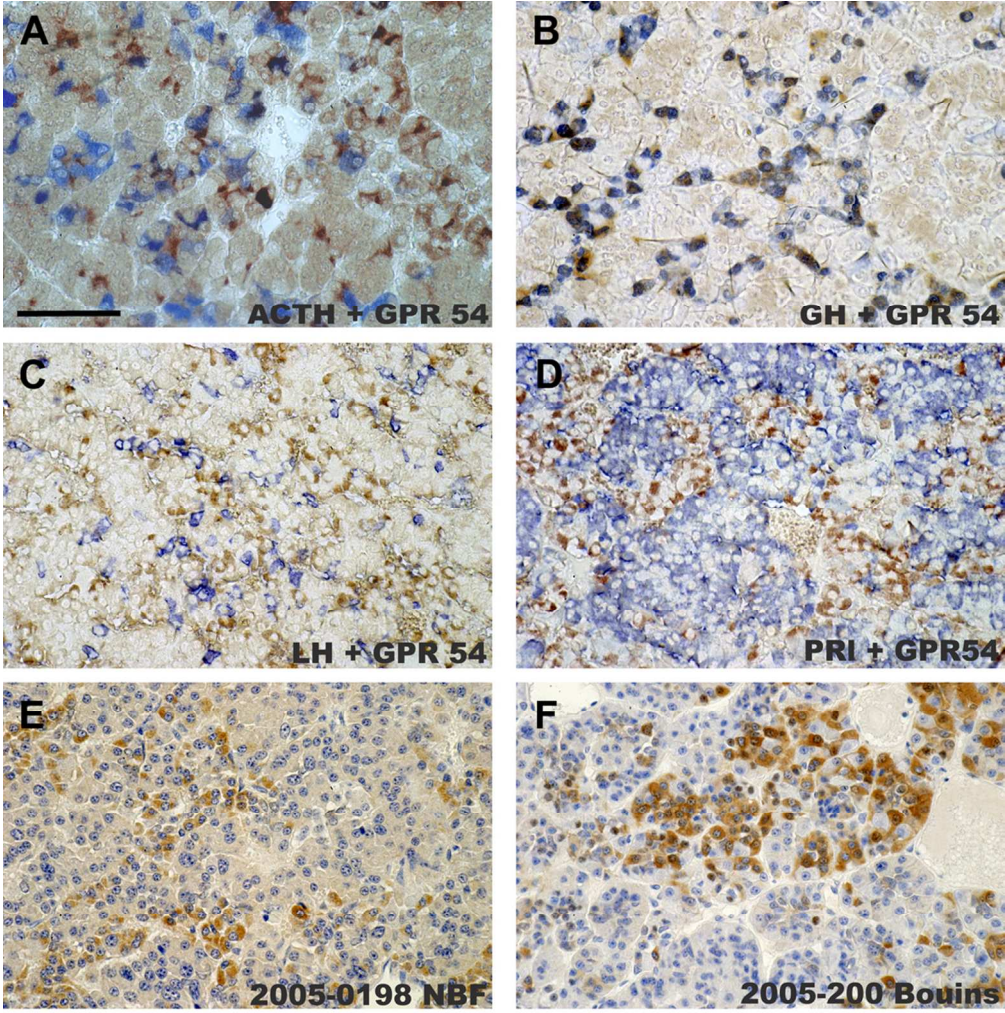


Figure 4





99x101mm (300 x 300 DPI)

Table 1.

	Control	Peptide-234	P Value
Mean GH (ng/ml)			
Pre Infusion	3.99 ± 1.33	4.42 ± 1.33	0.84
Infusion	4.49 ± 1.49	14.36 ± 2.15	<0.005
Post Infusion	5.93 ± 1.20	8.94 ± 1.13	0.11
Mean LH (ng/ml)*			
Pre Infusion	2.70 ± 0.34	2.73 ± 0.43	0.96
Infusion	2.44 ± 0.25	2.89 ± 0.55	0.50
Post Infusion	2.60 ± 0.11	1.78 ± 0.34	<0.05
Mean prolactin (ng/ml)*			
Pre Infusion	27.89 ± 9.21	58.67 ± 14.59	0.12
Infusion	17.02 ± 7.34	44.91 ± 15.87	0.16
Post Infusion	5.58 ± 3.23	27.74 ± 9.23	0.06
Mean cortisol (ng/ml)*			
Pre Infusion	11.90 ± 2.79	8.08 ± 1.96	0.31
Infusion	8.51 ± 1.81	8.26 ± 1.55	0.92
Post Infusion	11.57 ± 2.00	18.45 ± 5.98	0.32

Table 1. Summary of plasma GH, LH, prolactin and cortisol from Experiment 1.

* data derived from experiments in Roseweir et al. (Roseweir et al. 2009).